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Targeting Gene Therapy to Cancer: A Review

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In recent years the idea of using gene therapy as a modality in the treatment of diseases other than genetically inherited, monogenic disorders has taken root. This is particularly obvious in the field of oncology where currently more than 100 clinical trials have been approved worldwide. This report will summarize some of the exciting progress that has recently been made with respect to both targeting the delivery of potentially therapeutic genes to tumor sites and regulating their expression within the tumor microenvironment. In order to specifically target malignant cells while at the same time sparing normal tissue, cancer gene therapy will need to combine highly selective gene delivery with highly specific gene expression, specific gene product activity, and, possibly, specific drug activation. Although the efficient delivery of DNA to tumor sites remains a formidable task, progress has been made in recent years using both viral (retrovirus, adenovirus, adeno-associated virus) and nonviral (liposomes, gene gun, injection) methods. In this report emphasis will be placed on targeted rather than high-efficiency delivery, although those would need to be combined in the future for effective therapy. To date delivery has been targeted to tumor-specific and tissue-specific antigens, such as epithelial growth factor receptor, c-kit receptor, and folate receptor, and these will be described in some detail. To increase specificity and safety of gene therapy further, the expression of the therapeutic gene needs to be tightly controlled within the target tissue. Targeted gene expression has been analyzed using tissue-specific promoters (breast-, prostate-, and melanoma-specific promoters) and disease-specific promoters (carcinoembryonic antigen, HER-2/neu, Myc-Max response elements, DF3/MUC). Alternatively, expression could be regulated externally with the use of radiation-induced promoters or tetracycline-responsive elements. Another novel possibility that will be discussed is the regulation of therapeutic gene products by tumor-specific gene splicing. Gene expression could also be targeted at conditions specific to the tumor microenvironment, such as glucose deprivation and hypoxia. We have concentrated on hypoxia-targeted gene expression and this report will discuss our progress in detail. Chronic hypoxia occurs in tissue that is more than 100–200 μm away from a functional blood supply. In solid tumors hypoxia is widespread both because cancer cells are more prolific than the invading endothelial cells that make up the blood vessels and because the newly formed blood supply is disorganized. Measurements of oxygen partial pressure in patients' tumors showed a high percentage of severe hypoxia readings (less than 2.5 mmHg), readings not seen in normal tissue. This is a major problem in the treatment of cancer, because hypoxic cells are resistant to radiotherapy and often to chemotherapy. However, severe hypoxia is also a physiological condition specific to tumors, which makes it a potentially exploitable target. We have utilized hypoxia response elements (HRE) derived from the oxygen-regulated phosphoglycerate kinase gene to control gene expression in human tumor cells in vitro and in experimental tumors. The list of genes that have been considered for use in the treatment of cancer is extensive. It includes cytokines and costimulatory cell surface molecules intended to induce an effective systemic immune response against tumor antigens that would not otherwise develop. Other inventive strategies include the use of internally expressed antibodies to target oncogenic proteins (intrabodies) and the use of antisense technology (antisense oligonucleotides, antigenes, and ribozymes). This report will concentrate more on novel genes encoding prodrug activating enzymes, so-called suicide genes (Herpes simplex virus thymidine kinase, *Escherichia coli* nitroreductase, *E. coli* cytosine deaminase, thymidine phosphorylase, cytochrome P450 isoforms, deoxycytidine kinase, and our initial work on the *Clostridium acetobutylicum* hydrogenase and flavodoxin), and their prospective prodrugs. Details of our work on placing the gene encoding cytosine deaminase under hypoxia control will be discussed.

Key words: Delivery; Tissue specific; Gene expression; Regulation; Enzyme prodrug therapy

The original goal of gene therapy was to correct a genetic disorder by inserting a functional gene into an organism to replace a defective one. More recently the concept of using gene therapy in the treatment of diseases other than inherited, single gene disorders has emerged. Over 100 clinical trials for gene therapy of cancer have already started, and early Phase I results have been published. Following initial cell-marking and feasibility studies, strategies currently under clinical investigation are the enhancement of tumor immunogenicity by insertion of cytokine or costimulatory molecule encoding genes, or direct tu-

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[2]Abbreviations used: HSV, herpes simplex virus; Mo-MuLV, Maloney murine leukemia virus; scFv, single chain antigen binding protein; EGF-R, epidermal growth factor receptor; CTLs, cytotoxic T cells; 5-FC, 5-fluorocytosine; 5-FU, 5-fluorouracil; AFP, alpha-fetoprotein; HSVtk, herpes simplex virus thymidine kinase; GCV, ganciclovir; PSA, prostate-specific antigen; CMV, cytomegalovirus; β-gal, β-galactosidase; CEA, carcinoembryonic antigen; t-PA, tissue-type plasminogen activator; HRE, hypoxia response element; PD-ECGF, platelet-derived endothelial cell growth factor; 5′-DFUR, 5′-deoxy-5-fluorouridine; gpt, guanine phosphoribosyl transferase; 6TX; 6-thioxanthine.

mor kill by insertion of tumoricidal genes, tumor suppressor genes, and genes encoding prodrug activating enzymes. The first gene therapy protocol for cancer treatment was approved in May 1989. Cancer patients with advanced melanoma were treated by reinfusion with their own tumor-infiltrating lymphocytes, which were transduced with a marker gene ex vivo (1). Treatment with these gene-modified lymphocytes was more effective than treatment with lymphokine-activated killer cells; effective tumor remission with gene therapy was achieved in 40% of melanoma patients.

The problem with cancer therapy is selectivity and specificity. It is relatively easy to design a cell cytotoxin that kills tumor cells in vitro. However, in order to only kill tumor cells in vivo and at the same time spare normal tissue, a very selective strategy is necessary. The effective current therapies, such as ionizing radiation, rely on spatial delivery and the increased proliferation rate of tumors in order to differentiate them from normal tissue. Some cancer gene therapy strategies rely on the same principle, but other means of selectively targeting tumor cells have also been developed that will add to tumor specificity. Cancer gene therapy can combine highly specific gene delivery with highly specific gene expression. Advances in gene delivery have been made using viral and nonviral methods, but effective and selective delivery of DNA to tumor cells remains a complex task due to a poor and disorganized blood supply, and high interstitial fluid pressure. In this report, we will concentrate on targeted rather than high-efficiency delivery (e.g., tissue-specific targeting or disease-specific targeting).

Another level of specificity can be added to selective delivery by targeting gene expression. Transcriptional promoters that are specifically functional in single tissues, or are active in specific disease states, or are induced by tumor-specific conditions have been identified during basic research into cancer progression and can be utilized for targeted expression.

Three basic approaches in the design of therapeutic genes for cancer have been undertaken: molecular chemotherapy, genetic immunopotentiation, and mutation compensation. Molecular chemotherapy uses the delivery of a toxin gene (so-called suicide genes) to tumor cells for eradication, genetic immunopotentiation boosts the immune system to reject the tumor, and mutation compensation either ablates activated oncogenes or induces tumor suppressor gene expression. In this report we have chosen to concentrate on the first strategy. Two therapeutic genes derived from micro-organisms have been tested extensively in vitro and in vivo, and Phase 1 clinical trials using the herpes simplex virus thymidine kinase and cytosine deaminase encoding genes are under way. Other prodrug activating enzymes are emerging in parallel to the development of new prodrugs for gene-directed enzyme prodrug therapy, and we will discuss these promising new strategies.

TARGETED DELIVERY

Advances have been made in the transfer of foreign genetic material to human cells, and high-efficiency delivery is now possible. However, targeted rather than highly efficient delivery is required for selective cancer gene therapy. Extracellular receptors and cell surface molecules specific to tumor cells have been identified that can be used to target delivery of therapeutic genes. Both viral and nonviral delivery vehicles have been developed for this purpose, including retro- and adenovirus, liposomes, polylysine constructs, T-cells, and even bacteria.

One of the original delivery methods was based on retroviral transduction, as they only infect dividing cells and can therefore be targeted broadly to proliferating cancer cells. Retrovirus constructs containing cytokine encoding genes were shown to inhibit metastasis formation and thereby reduce the tumor burden in experimental animals (2). Retroviral vectors have been developed further to achieve selective transfection.

To target only actively dividing cells a recombinant herpes simplex virus (HSV^2) vector was constructed that lacked the ribonucleotide reductase encoding gene (3). Ribonucleotide reductase is only expressed in actively dividing cells and is essential for viral replication; the recombinant vector therefore has to rely on the host cell for the generation of deoxyribonucleotides for DNA synthesis. The recombinant HSV vector carrying the *lacZ* marker was shown to replicate in and kill only dividing cells in vitro. *lacZ* activity was observed only in tumor nodules in the liver following intrasplenic injection of the colon carcinoma cell line HT29 into nude mice, followed a week later by HSV vector intrasplenic injection. Only minimal *lacZ* activity was seen in normal liver.

Targeted Delivery: Retroviral Vectors With Modified Envelope Proteins

The transfer of genes by retroviral vectors is entirely dependent on the interaction between proteins in the viral envelope and suitable counterreceptors on the target cell surface (4). The envelope (env) protein of the Moloney murine leukemia virus (Mo-MuLV), on which most retroviral vectors are based, is made up of two covalently linked peptides, termed SU and TM. The amino-terminal SU peptide mediates binding of the virus to its cell surface receptor whereas the carboxy TM domain anchors the molecule to the viral membrane. Retrovirus can be targeted specifically to cells expressing a particular surface protein if the SU domain of the viral env protein is replaced by a single chain antigen binding protein (termed scFv) (5–7). scFv consists of the variable regions of both heavy and light chains of an antibody molecule. However, only a small subset of surface proteins can function as appropriate receptors for the uptake and expression of these genetically engineered retroviral

vectors. Therefore, a means of rapidly identifying differentially expressed cell surface molecules that can function as receptors for these retroviral vectors was developed (8). Currently the vectors are being used to screen panels of monoclonal antibodies raised against vascular endothelial cells.

Targeted Delivery: Epidermal Growth Factor Receptor to Target Lung Cancer Cells

The epidermal growth factor receptor (EGF-R) is overexpressed in the majority of lung cancer cells. Human heregulin is a ligand for EGF-R (and the related receptor HER-2/neu) and was therefore used in the design of a retroviral vector to target delivery to lung cancer cells (9). By replacing the Mo-MuLV envelope with heregulin encoding sequences the ecotropic virus was able to cross the species barrier and infect EGF-R overexpressing human cells. Human cells that showed low EGF-R expression were resistant to the viral infection.

Alternatively, recombinant human epithelial growth factor was incorporated into a polylysine vector to target cells rich in EGF-R (10). In the presence of replication defective adenovirus, which was required as an endosomal lysis agent, the EGF-DNA complex was able to transfect lung cancer cells at a high efficiency in vitro, whereas colon carcinoma cells showed little DNA uptake.

Another strategy targets cytotoxic T cells (CTLs) to tumor cells that express EGF-R (11). A recombinant T-cell receptor was constructed consisting of a scFv derivative of an anti-EGF-R monoclonal antibody, which serves as the extracellular antigen binding domain, and the zeta chain of the TCR/CD3 complex, which serves as a signal transduction domain. A hinge region between the binding and signal transduction domain appeared to be required for efficient binding. In vitro cross-linking of the recombinant receptor with externally added EGF-R in transfected T cells showed that the signal transduction pathway was functional. Addition of these transfected CTLs to tumor cells in culture efficiently lysed EGF-R positive tumor cells.

Targeted Delivery: Folate Receptor to Target Cancer Cells

Folate receptors are overexpressed in the majority of tumors. Folate receptors are internalized through caveolae in a process termed potolysis, which is different from endocytosis. This process could be exploited for the targeted delivery of DNA by conjugating folate to poly-L-lysine (12,13). Although marker gene expression was moderate, it was specific to tumor cells that highly overexpress the folate receptor and could be improved by the addition of replication defective adenovirus.

Targeted Delivery: c-kit Receptor to Target Hematopoietic Cells and Lung Cancer

The proto-oncogene c-*kit*, a receptor tyrosine kinase, and its ligand, Steel factor, are required for melanogenesis, hematopoesis, and gametogenesis, and also play a role in cell-matrix adhesion (14). c-*kit* is highly expressed on hematopoietic progenitor and small-cell lung cancer cells. A delivery vehicle was constructed to target hematopoietic progenitor cells consisting of DNA condensed with polylysine covalently linked to streptavidin, the biotinylated ligand Steel factor (because streptavidin binds biotin), and polylysine-coated adenovirus for endosomal lysis (15). Only c-*kit*-positive cells were transfected. The streptavidin/biotin system is versatile and could be changed by the addition of other biotinylated ligands.

Targeted Delivery: Anaerobic Bacteria to Target Solid Tumors

Intravenously injected clostridial spores specifically locate to solid tumors where they germinate in necrotic and hypoxic regions (16). In order to improve the efficacy of this unconventional treatment, the cytosine deaminase [an enzyme able to convert 5-fluorocytosine (5-FC) to the toxic anticancer drug 5-fluorouracil (5-FU), discussed in detail later] encoding gene from *Escherichia coli* was transfected into the nonpathogenic anaerobe *Clostridium beijerinickii* (17). Supernatant containing active enzyme taken from the transformed bacteria could sensitize murine carcinoma cells 500-fold to 5-FC in air. As an alternative, the *E. coli* nitroreductase gene (which activates CB 1954 to a bifunctional alkylating agent, more later) was introduced into *C. beijerinickii* and the resultant spores injected into tumor-bearing mice (18). Tumor lysates were tested and shown to contain the nitroreductase enzyme.

TARGETED EXPRESSION

Not only delivery can be targeted, but also gene expression to further improve selectivity of gene therapy of cancer. Therefore, even if therapeutic genes are delivered to normal tissue, the regulatory sequences should prevent them from being expressed. Based on extensive basic research, genes have been identified that are expressed in either a tissue-specific or cancer-specific way, or that are induced by conditions specific to tumors. The regulatory sequences required for the selected expression can lie within the basic promoter or, in the case of enhancer sequences, up to several hundred bases upstream or downstream. Isolated enhancer elements can function on their own or in combination with other regulatory sequences, and in both orientations and almost any location to the gene of interest.

Tissue-Targeted Expression

Tissue-Targeted Expression: Alpha-Fetoprotein to Target Hepatocellular Carcinoma. Although alpha-fetoprotein (AFP) is normally only expressed in fetal liver and not in adult tissue, it has been found to be abnormally activated in hepatocellular carcinoma. Transgenic mice carrying the SV40 large T-antigen develop hepatocellular carcinoma (19). Crossing

these mice with mice transgenic for HSV thymidine kinase [HSVtk activates the antiviral agent ganciclovir (GCV) to a toxic drug, discussed in detail later] gene under the control of the AFP promoter delayed tumor progression significantly when treated with GCV.

Adenoviral delivery of the HSVtk encoding gene under the AFP promoter/enhancer control also sensitized specifically AFP expressing human hepatocellular carcinoma cells to GCV in vitro and in vivo (20,21). A short region (0.3 kb) of the human AFP gene promoter was found to be sufficient to direct expression of the HSVtk gene specifically to hepatoma cells (22). Gene expression could be modulated and toxicity increased further by treating the transfectants with dexamethasone due to a glucocorticoid response element in the AFP promoter. AFP-regulated wt p53 expression was equally restricted to AFP-producing cells and could inhibit cell growth in vitro and increase sensitivity to cisplatin (23).

However, disturbing results were reported when adenoviral delivery of a construct containing the AFP promoter and a marker gene was found to preferentially locate in normal liver of experimental animals, instead of the hepatoma (24). Another report also described extensive liver damage following adenoviral delivery of HSVtk, which questions the use of adenovirus as a delivery vehicle (25).

Tissue-Targeted Expression: Prostate-Specific Antigen to Target Prostate Cancer. Prostate-specific antigen (PSA) is a serine protease involved in the degradation of the major proteins in the seminal coagulum leading to semen liquefaction [for a review see (26)]. PSA is produced preferentially in the prostate and is often overexpressed in prostate cancer. However, PSA has also been found in accessory male sex glands, breast cancer, and milk of lactating women.

The upstream region (620 bp) of the PSA encoding gene was used to specifically drive expression of a marker gene in PSA^+ prostate cell lines (27). Expression could not be detected in nonprostate and PSA^- cells, and could be stimulated further by dihydrotestosterone in PSA^+ cells. The transfected construct showed competitive inhibition of the endogenous genomic promoter, indicating that prostate-specific DNA binding proteins are required to activate the PSA promoter. By adding the strong nonspecific cytomegalovirus (CMV) promoter to the PSA promoter to drive marker gene expression, tissue specificity could be retained and a further four- to fivefold increase in expression achieved. It therefore appears that the PSA promoter contains negative regulatory elements that can override the promiscuous expression of the CMV promoter to limit expression to PSA^+ cells. Further analysis of the PSA promoter revealed both a proximal and a distant promoter that are required for enhanced expression (28). The distant 822-bp regulatory sequence increased the otherwise low expression of the proximal promoter, enhanced responsiveness to dihydrotestosterone, and retained tissue specificity. The distant promoter on its own, however, showed only low activity.

The PSA promoter was also used to direct expression of antisense DNA to polymerase α and topoisomerase IIα to prostate cancer cells (29). Varying levels of expression were detected in prostate cancer cells, but none in the nonprostate cells. Lipofection-mediated transfer of either of the two antisense constructs reduced growth in vitro, which could be increased to 55% by cotransfection of both antisense constructs.

Tissue-Targeted Expression: von Willebrand Factor to Target Endothelial Cells. Angiogenesis, the sprouting of new blood vessels from the preexisting vessel bed, is an absolute requirement for the growth of tumors beyond the size of a mm^3. Tumor vasculature is an attractive target for therapy, because about 1000 tumor cells rely on every endothelial cell for the delivery of nutrients. Not all endothelial cells need to be damaged to achieve tumor response; effecting only a few can shut down the blood supply to the entire tumor. As a first step towards antiangiogenic gene therapy, the von Willebrand factor (vWf) promoter was analyzed to target the tumor vasculature (30). A region that encompasses most of the first noncoding exon showed strong promoter activity, which appeared to be endothelial cell specific. Growth suppression in vitro was evident when the vWf promoter was used to drive expression of HSVtk followed by GCV treatment.

Endothelial cell-specific genes have been identified using differential display (31). A receptor tyrosine kinase, tie-2/tek, was identified that is only expressed in endothelial cells where it is upregulated during neovascularization. The tie-2/tek promoter is currently being evaluated for vascular-targeted gene therapy.

Tissue-Targeted Expression: DF3 (MUC1) Promoter to Target Breast Cancer. DF3 (MUC1) is a high molecular weight mucin-like glycoprotein that is overexpressed in the majority of breast cancers. By fusing the DF3 promoter to the coding region of HSVtk it was possible to selectively sensitize DF3-positive breast tumor cells to GCV (32). It was further shown that IP injection of defective recombinant adenoviral vectors containing the DF3-HSVtk constructs into animals with breast cancer metastasis followed by GCV treatment inhibited tumor growth (33).

Tissue-Targeted Expression: Albumin Enhancer to Target Liver Cancer. The albumin enhancer element and promoter were used to exclusively target expression of the marker gene β-galactosidase (β-gal) to hepatoma cells (34). Delivery in vivo was performed by injection of an ecotropic retroviral vector containing the liver-specific expression system. Expression could only be detected in dividing hepatocytes following injection into the spleen or liver, not in other tissue or in nondividing cells.

Tissue-Targeted Expression: Tyrosinase Promoter to Target Melanoma Cells. The tyrosinase enzyme is part of the pigment producing pathway in skin and mela-

noma cells. The 5′ region of the tyrosinase encoding gene or the tyrosinase-related protein were used to direct expression of the marker gene β-gal specifically to human and murine melanoma cells and melanocytes in vitro (35). Injection of this construct into tumor-bearing mice showed expression only in the melanoma cell line B16-derived tumors and in some normal melanocytes, but not in COLO26 tumors or other normal surrounding tissue. To develop the system further the genes encoding either IL-2, IL-4, or macrophage colony-stimulating factor (M-CSF) were placed under the control of the murine tyrosinase 5′ region (36). Only IL-2-expressing B16 transfectants were prohibited to grow in syngeneic mice. However, injection of the DNA construct into established tumors did not effect tumor growth, even though expression of the cytokine genes could be detected. Expression of the HSVtk encoding gene under the tyrosinase promoter control following delivery by retrovirus and GCV treatment could reduce lung metastasis of B16 cells in syngeneic mice (37). The magnitude of reduction indicated a significant bystander effect, which could not be detected in immunodeficient mice. It therefore appears that the bystander effect of the HSVtk system requires an immune component.

Tissue-Targeted Expression: Myelin Basic Protein Promoter to Target Glioma Cells. The marker gene β-gal was placed under the control of either the mouse glial fibrillary acidic protein promoter (GFAP), the myelin basic protein promoter (MBP), or the myelin proteolipid protein promoter (PLP) in order to target expression to glioma cells (38). Expression in vitro was restricted to glioma cells, as transduced fibroblasts and myeloma cells did not show any β-gal activity. The MBP promoter region showed the strongest promoter activity and was therefore used to direct expression of the HSVtk encoding gene. Subsequent treatment of the transduced cells with GCV specifically killed the glioma cells in vitro.

Tissue-Targeted Expression: Osteocalcin Promoter to Target Osteosarcoma. The noncollagenous bone matrix protein osteocalcin is expressed at high levels in osteoblasts. The osteocalcin promoter could specifically drive expression in osteoblasts in vitro, and osteocalcin-regulated HSVtk expression resulted in osteosarcoma tumor regression in vivo following nucleoside treatment (39).

Disease-Targeted Expression

Disease-Targeted Expression: Carcinoembryonic Antigen Regulatory Sequence. Human carcinoembryonic antigen (CEA) is a member of a family of membrane glycoproteins that are overexpressed in many carcinomas; in 40% of patients with gastric cancer CEA levels are increased. CEA functions as a homotypic intercellular adhesion molecule in vitro (40).

The promoter for CEA was used to control gene expression of the HSVtk encoding gene within CEA-positive pancreatic carcinomas (41) or lung cancer cells (42). Only CEA-positive cells expressed the transgene, whereas negative ones did not. A reduction in tumor size of xenografts derived from the transfectants could be achieved by treating the mice with GCV, even if only 10% of the tumor cells contained the HSVtk gene.

Alternatively, the CEA transcriptional regulatory sequence was fused to the cytosine deaminase encoding gene to selectively sensitize colorectal carcinoma cells to 5-FC (43). Following retroviral delivery the chimeric genes were expressed only in CEA-positive cells. Studies using human tumor xenografts showed that the cytosine deaminase/5-FC combination in solid tumors could generate complete cures if only 4% of the solid tumor cell mass expressed this enzyme.

The sequences controlling CEA expression have been analyzed. Four *cis*-acting elements were mapped to a positive regulatory region and one element to a silencing region (40). Several nuclear factors were identified that bind to the regulatory regions: USF, Sp1, and Sp1-like factor. Independently, sequences containing the promoter, sequences essential for basic expression, copy number regulation, and selective expression were identified (44). The optimum combination of these regulatory sequences results in a two- to fourfold increase in expression over that induced by the strong SV40 promoter in CEA-positive cells.

Disease-Targeted Expression: HER-2/neu (erb B2). The oncogene HER-2/neu is overexpressed in about one third of breast and pancreatic tumors, and is involved in the transformation of prostatic epithelial cells. Overexpression involves transcriptional upregulation with or without gene amplification. HER-2/neu is a transmembrane glycoprotein related to EGF-R that functions as a growth factor receptor to regulate cell growth and transformation. The HER-2/neu promoter was used to target expression of cytosine deaminase selectively to HER-2/neu$^+$ cells, whereas no enzyme could be detected in HER-2/neu$^-$ cells (45). Significant cell killing was observed in the HER-2/neu$^+$ transfectants when treated with 5-FC, whereas HER-2/neu$^-$ transfectants were resistant to the drug.

Disease-Targeted Expression: Myc-Max Response Elements. The c-*myc* proto-oncogene has been implicated in the control of cell proliferation, differentiation, and apoptosis. The Myc family of proteins, including c-*myc*, can form heterodimers with the Max protein, which specifically binds to its recognition sequence to activate transcription (46). The binding sequences for the Myc-Max heterodimer can direct gene expression to cells that overexpress c-*myc*. Four repeats of the Myc-Max response element were ligated to the HSVtk encoding gene in order to target gene expression to a range of *myc*-overexpressing cancer cells, including small-cell lung cancer cells and colon carcinoma cells (47,48). Transfected tumor cells exhibited increased sensitivity to GCV in vitro and liposomal delivery to established tumors followed by drug treatment induced tumor regression in vivo.

Condition-Targeted Expression

Condition-Targeted Expression: Early Growth Response-1 Gene Regulated by Ionizing Radiation. The early growth response gene (*Egr-1*) is a ubiquitous immediate early gene that encodes a zinc finger transcription factor. *Egr-1* is induced by a wide variety of stimuli including ionizing radiation and cytokines. However, it is expressed only at low levels in many human tumor cells (49). Still, the radiation controllability of *Egr-1* has been exploited to regulate gene expression for cancer therapy. By linking the radiation-inducible CArG elements of the *Egr-1* promoter to the TNF-α encoding gene, expression could be regulated by ionizing radiation in a temporal and localized way (50). Injection of *Egr*-TNF transfected hematopoietic cells into xenografts grown from radiation-resistant squamous cell carcinoma followed by radiation increased tumor cures compared to radiation alone. Alternatively, direct intratumoral injection of liposomes or adenoviral delivery of the *Egr-1*-controlled TNF construct into the radiation- and TNF-resistant tumors followed by 20 or 50 Gy of radiation could significantly reduce tumor size (51,52). The effect on tumor growth can be partly attributed to damage of the tumor vasculature following treatment (53). Adenoviral delivery of the *Egr*-TNF construct and radiation resulted in extensive intratumoral vascular thrombosis and necrosis. No thrombosis was detected in treated normal tissue, indicating that a tumor-specific occlusion of microvessels was induced. The *Egr-1* control element was also utilized to direct radiation-controlled expression of HSVtk to glioma cell lines (54).

Condition-Targeted Expression: Tissue-Type Plasminogen Activator Regulated by Radiation. Plasminogen activators have clinical significance as thrombolytic agents for management of stroke and myocardial infarction. Tissue-type plasminogen activator (t-PA) is induced over 50-fold after irradiation with X rays in otherwise radioresistant human melanoma cells (55). The t-PA protease might have a function equivalent to the SOS repair system in prokaryotes. An X-ray-inducible element was identified in the t-PA promoter that might be useful for radiation-inducible gene therapy.

Condition-Targeted Expression: Regulation by Glucose-Regulated Protein GRP78/BiP Promoter. The expression of the glucose-regulated protein GRP78/BiP is strongly upregulated by tumor-specific conditions such as glucose deprivation, anoxia, and acidity. Elevated levels of GRP proteins protect the cell against stress, as they function as chaperones and calcium binding proteins (56). The promoter of the GRP78/BiP encoding gene was therefore used to control expression of a marker gene in a murine fibrosarcoma model (57). In vitro glucose deprivation of transduced fibrosarcoma cells showed an eightfold induction over nonstressed cells. Comparison with the viral SV40 promoter showed a twofold lower basal level of expression. Tumors grown from transduced cells showed central pockets of enhanced expression of the marker gene, indicating that in vivo stress response could control the glucose-regulated marker gene.

Condition-Targeted Expression: Hypoxia-Regulated Gene Expression. Our method is designed to take advantage of the abnormal physiology that exists in almost all solid tumors, regardless of their origin or location, and use this tumor-specific condition to control the expression of therapeutic genes (58).

Aggressive tumors often have insufficient blood supply, partly because tumor cells grow faster than the endothelial cells that make up the blood vessels, and partly because the newly formed vascular supply is disorganized (59). This results in areas of acidity and nutrient deprivation, as well as regions with reduced oxygen concentrations (hypoxia). Direct measurements taken in patients showed a median oxygen level in normal tissue of 24–66 mmHg (3.1–8.7% O_2), whereas those in tumors ranged from 10 to 30 mmHg (1.3–3.9%). More importantly, tumors routinely posses microregions with pO_2 levels of less than 2.5 mmHg (0.3%), levels at which cells are three times more resistant to radiation than aerated cells (60). Such hypoxia is not necessarily chronic. Blood vessels can open and close, creating microregions with acute hypoxia. It is important to note that cells in this aberrant environment can remain viable and are often chemo- and radioresistant. Hence, hypoxia is considered a major hindrance to therapy. In fact, a recent study of patients with cervical cancer showed the oxygen status of a tumor to be the single most important prognostic factor, ahead of age of patient, menopausal status, clinical stage, and size and histology of tumor (61). However, it is our aim to exploit the presence of this subpopulation of resistant cells.

Hypoxic conditions can modulate the expression of a number of genes including those encoding growth factors, transcription factors, and glycolytic and DNA repair enzymes [for a recent review see (62)]. Hypoxic expression is controlled by the binding of the transcription factor, hypoxia inducible factor-1 (HIF-1), to a short DNA sequence [hypoxia enhancer or hypoxia response element (HRE)], which can be situated at any distance and both orientations from the coding region. We have utilized the mouse phosphoglycerate kinase-1 HRE (63) in this study to control expression of marker and therapeutic genes in response to low oxygen in vitro and in vivo.

The use of hypoxia-responsive enhancer elements enabled the regulation of the marker gene CD2 in response to low oxygen conditions (58). These studies showed that the in vitro response was time and oxygen concentration dependent, with no protein induction at oxygen levels similar to those observed in normal tissue. The level of hypoxia that was sufficient to induce the CD2 marker gene in vitro has been clinically observed (61,64). Interestingly, not only hypoxia increased the levels of the marker gene, but subsequent reaeration increased it further. Although the reasons are not clear, it is possible that protein

synthesis is restarted following growth arrest during reoxygenation, or that oxygen radicals similar to those found in reperfusion injury induced gene expression.

When the transfected tumor cells were grown as xenografts, expression of the marker gene was confined to areas adjacent to necrosis. Hypoxic induction in vivo of the HRE-controlled CD2 was confirmed by combining CD2 staining with the comet assay. For the comet assay (65), tumor-bearing mice are treated with radiation (which causes single strand breaks preferentially in oxic cells) and a bioreductive drug (which induces DNA cross-links only in hypoxic cells). Single cell electrophoresis of single cells isolated from the treated tumors can differentiate the two populations. The hypoxic cells, according to the comet assay, stained positive for CD2, whereas the aerobic ones did not. These in vitro and in vivo results demonstrate the selectivity of the system and its potential for tumor-specific targeting of gene expression.

A recent article studied the possibility of delivering genes to hypoxic cells in rat hearts by injection or retroviral delivery (66). It showed that not only could foreign genes be taken up in ischemic/reperfused tissue, but that the genes were transcribed and the protein synthesis machinery of the injured cells could produce recombinant proteins that retained enzymatic activity. Gene delivery to regions of chronic hypoxia in solid tumors will remain a formidable task, but the regions we aim to infect are those of transient hypoxia. These regions contain blood vessels that open and close, potentially allowing delivery of the therapeutic genes by repeated application.

Regulation by Bacterial Regulatory Sequences

The luciferase reporter gene linked to a chimeric promoter containing binding sequences for the bacterial tetracycline repressor could be transactivated by cotransfection with genes encoding tTA (67). The addition of tetracycline could reduce luciferase expression of the tet repressor/tTA transfected cells in a graded manner. Enhanced tetracycline-regulated expression of HSVtk was achieved by incorporating the tet repressor/tTA system in a retroviral construct (68).

An interesting, though not targeted expression system, consists of the bacteriophage T7 promoter and T7 RNA polymerase encoding gene (69). This T7 system can initiate and maintain itself, and requires no cellular factors for transcription, and is therefore inclined to function in any mammalian system. By cotransfecting constructs containing the promoter-driving expression of a marker gene and its specific RNA polymerase, high cytoplasmic expression (up to 200-fold increase over nuclear gene expression) could be achieved in a wide variety of mammalian cells both in vitro and in vivo.

Alternative Splicing

A critical step in eukaryotic gene expression involves the removal of intervening sequences encoded by introns from pre-mRNA transcripts prior to their transport to the cytoplasm where translation occurs [for a recent review see (70)]. This process is termed "splicing." It is now appreciated that a large proportion of cellular genes encode primary transcripts in which exonic sequences can be spliced in different ways to generate multiple mRNA species that encode protein isoforms with unique structures and functions (71). This latter process, termed "alternative splicing," enhances the coding potential of the genome without the need to resort to gene duplication events. Alternative splicing is a highly regulated process with distinct patterns of splicing occurring during cellular differentiation, in different tissues, or in response to extracellular signals.

In the last year or so, we have begun to investigate the possibility that differences in the ability of normal and malignant cells to alternatively splice pre-mRNA transcripts could perhaps be exploited as a means of targeting therapeutic genes to the malignant population. Specifically, we have made a series of vectors in which the cytosine deaminase gene is fused in-frame to a cassette that contains two or more alternatively spliced exons derived from the gene encoding the adhesion protein CD44 such that expression of a functional CD44-cytosine deaminase chimera will only occur in cells that are capable of appropriately splicing out the intronic sequences that separate the CD44 exons (72). Although at an early stage of development, such vectors may allow the selective destruction of malignant and/or other rapidly proliferating cell types because these have been shown in numerous studies to differentially express certain alternatively spliced CD44 isoforms (73–75).

THERAPEUTIC GENES: MOLECULAR CHEMOTHERAPY

The best choice for enzymes for gene-directed enzyme prodrug therapy would be monomeric enzymes of viral or bacterial origin with a wide substrate specificity (76). It is therefore not surprising to see a viral (HSVtk) and a bacterial (cytosine deaminase) gene as the first candidates of therapeutic genes in Phase I clinical trials.

Prodrugs are defined as chemicals that, even at high concentrations, are nontoxic unless specifically activated by cellular conditions or enzymes to toxic metabolites. Using gene therapy it is possible to introduce a foreign enzyme-encoding gene into human cells in order to sensitize them to an otherwise nontoxic prodrug. The ideal activated drug should be at least 100-fold more cytotoxic than the parent compound, should easily diffuse to kill nontransfected bystander cells, and have a significant half-life to kill bystanders but not escape into the blood stream to cause systemic toxicity.

Herpes Simplex Virus Thymidine Kinase

HSVtk is the model for gene-directed enzyme prodrug therapy and has been used extensively in pre-

clinical studies. HSVtk converts nontoxic nucleoside analogues, such as GCV, to phosphorylated compounds that can act as chain terminators and specifically kill dividing cells. Specific expression of the suicide gene in dividing cells can be further targeted by using retroviral gene transfer. Direct injection of packaging cells producing retrovirus containing the HSVtk encoding gene into established macroscopic liver metastasis in rats followed by GCV treatment resulted in regression of tumor volume and reduction of mean cell mass by 60-fold (77). HSVtk/GCV was also able to suppress metastasis in the short and long term in a mouse model of prostatic cancer, even after regrowth of the primary tumor (78).

Transgenic mice carrying the rat *neu* oncogene under the control of the mouse mammary tumor virus LTR develop breast cancers within 2–3 months of birth. Crossing these *neu* mice with mice transgenic for the HSVtk gene under the control of its own promoter and the polyoma enhancer resulted in double transgenic mice that still developed tumors at the same rate as the *neu* mice (79). Intratumorally, treatment with GCV reduced tumor growth in the double transgenics, but had no effect on the *neu* single transgenic mice. However, mammary tumors induced by the activated rat *neu* oncogene could not be eradicated by retroviral delivery of HSVtk followed by GCV treatment (80). The lack of success could be attributed to the combination of low transduction efficiency by the retroviral vector and the lack of a bystander effect in the breast cancer cells due to the absence of functional gap junctions.

It has previously been shown that only a fraction of cells needed to contain the gene in order to activate the prodrug to its toxic product and kill untransfected bystanders. Gap junctions, which play a role in intercellular communications and require cell contact, had been implicated in this bystander effect. HeLa cells show very little ability to communicate via gap junctions, and also demonstrate little or no bystander effect when transfected with HSVtk and treated with GCV (81). However, the bystander effect could be restored to HeLa cells by transfection with the gene encoding the gap junction protein connexin 43. This strongly suggests that the bystander effect of the HSVtk/GCV system depends on gap junctions. However, not all systems displaying a bystander effect rely on gap junctions.

Verapamil, a calcium channel antagonist that is also used to inhibit multidrug resistance, was shown to protect bystander cells from the HSVtk/GCV system (82). Verapamil inhibits the bystander effect both in coculture in vitro and also in a tumor model, but is ineffective if given more than 2 days after GCV treatment. No difference in sensitivity of the transfectants was observed following verapamil and GCV treatment.

To address the effect of the immune system on treatment, rat mesothelioma cell lines transduced with HSVtk were grown in the flanks of either Fisher rats, nude rats, or Fisher rats immunosuppressed with cyclosporin (83). Adenoviral delivery of HSVtk followed by GCV treatment was significantly more effective in the nude or immunocompromised rats compared to the normal Fisher rats, indicating that the immune response against adenovirally transduced cells limited efficacy. Hence, it implied that immunosuppression might be a useful adjunct in this system.

However, the immune system is essential for an efficient bystander effect. Neighboring untransfected tumor cells could be killed by inoculating syngeneic transfected cells and even irradiated xenogeneic transfected cells into tumor-bearing mice (84). Following GCV treatment upregulation of the cytokines IL-1α and IL-6 was evident as well as widespread necrosis, which resulted in prolonged animal survival.

Current work is under way to optimize the activity of HSVtk to reduce the amount of GCV necessary (85). By random mutagenesis of the HSVtk encoding gene new mutants were identified that induced a 43-fold greater sensitivity to GCV in mammalian cells.

A Phase 1 clinical trial is currently under way to test adenoviral delivery of HSVtk to patients (86). Patients with advanced brain tumors will receive stereotactic-guided injection of the vector construct at escalating doses followed by systemic GCV treatment. However, high toxicity has since been reported in mice after IV injection of adenovirus carrying the HSVtk followed by GCV treatment (25). HSVtk was found to accumulate in the liver, causing extensive liver degradation. The toxicity was prevalent for at least 7 weeks after vector administration. A mechanism of toxicity of phosphorylated GCV, which is independent of cell division, probably exists as the resting liver is nonproliferating.

Cytosine Deaminase

Mammalian cells do not produce significant amounts of the enzyme cytosine deaminase. *E. coli*, however, possess the gene encoding cytosine deaminase and are capable of converting cytosine to uracil. The introduction of the bacterial gene to mammalian cells can sensitize them to the prodrug 5-FC, which is converted to the cytotoxic agent 5-FU. 5-FU is currently the most effective single agent for the treatment of colorectal carcinoma. The human colorectal carcinoma cell line WiDr could be sensitized a 1000-fold to 5-FC by the introduction of the cytosine deaminase encoding gene (87). In vivo experiments showed that the half-life of 5-FC is 40 min in nude mice. Daily treatment for 10 days of tumor-bearing animals with 500 mg/kg 5-FC could reduce tumor size and thymidine incorporation of the transfected tumors. Interestingly, however, 5-FU had little effect on these tumors.

Significant amounts of 5-FU were found in the supernatant of cytosine deaminase transfected cells treated with 5-FC, which would account for the bystander effect observed (88). However, uptake studies with radiolabeled 5-FC indicated that moderate and unsaturatable amounts of the drug accumulated

intracellular, possibly by diffusion only. 5-FC uptake is therefore a bottleneck in this treatment strategy.

5-FU is also used as a radiation enhancer. Cell kill in vitro could be enhanced by treating cytosine deaminase transfected cells with a combination of 5-FC and radiation (89). A bifunctional fusion protein consisting of cytosine deaminase and HSVtk was constructed (90). The protein showed enzyme activity for both enzymes, increased radiosensitivity and a slight synergistic effect when the transfectants were treated with both 5-FC and GCV.

A comparison of HSVtk/GCV and cytosine deaminase/5-FC in a human colorectal xenograft showed that the HSVtk/GCV system required more than 10% of the cells to be transfected for an antitumor effect (91). However, less than 4% of transfected cells in the cytosine deaminase/5-FC system were sufficient to achieve 60% cure rate. The lack of a bystander effect in the HSVtk/GCV system could be explained by transmission electron microscope analysis of the tumor cells, which showed that although they contained desmosomes, they lacked gap junctions. The reliance of the HSVtk/GCV system on functional gap junctions may limit its use in the clinic.

We have utilized the cytosine deaminase encoding gene in our hypoxia-regulated system (58). The gene was placed under the transcriptional control of the 9-27 gene promoter into which was inserted a triplet of HRE derived from the PGK gene. Human tumor HT1080 cells transfected with this DNA construct exhibited a sevenfold increase in enzyme activity following hypoxia compared to normoxia. The increase in enzyme activity resulted in a 5.4-fold increase in sensitivity of the transfectants to 5-FC following hypoxia compared to normoxia. This suggested that 5-FC had been converted to the toxic 5-FU by cytosine deaminase following the hypoxic exposure.

Cytochrome P450 Enzymes (CYP450 2B1)

Liver cytochrome P450 enzymes are able to convert cyclophosphamide to a potent alkylating agent that has antitumor activity. However, the therapeutic effectiveness is limited by hematopoietic, renal, and cardiac cytotoxicity. The transfer of these liver enzymes to tumor cells would reduce systemic toxicity and increase antitumor activity. The cytochrome P450 isomer, CYP2B1, converts cyclophosphamide to a hydroxy intermediate, which brakes down to form the DNA alkalyting toxin phosphoramide mustard. Gliosarcoma cells transfected with the gene encoding CYP2B1 showed increased sensitivity to cyclophosphamide (92–94). The cells could be protected against the toxic effect by treatment with the CYP2B1 inhibitor metyrapone. Treatment with cyclophosphamide or ifosphamide of a mixed culture consisting of 10% transfected cells showed a bystander effect that did not require cell–cell contact. Fischer rats implanted with CYP2B1-expressing tumor cells showed an increased sensitivity to cyclophosphamide treatment; a significant growth inhibition was seen in the CYP2B1-positive tumors compared to a modest delay in CYP2B1-negative tumors following treatment.

Platelet-Derived Endothelial Cell Growth Factor/ Thymidine Phosphorylase

Platelet-derived endothelial cell growth factor (PD-ECGF) functions as a chemotactic and angiogenic growth factor, and is used as a prognostic indicator in a range of tumor types. Expression of PD-ECGF is on average 27-fold increased in human breast tumors compared to normal breast (95). The level of PD-ECGF in the tumor cell line MCF-7 is similar to that of the low expressers in human breast tumor biopsis. PD-ECGF is identical to the enzyme thymidine phosphorylase (dThdPase), which catalyzes the phosphorolytic cleavage of 5′-deoxy-5-fluorouridine (5′-DFUR) to the anticancer agent 5-FU. Transfection of MCF-7 cells with the PD-ECGF encoding gene increased the cells' sensitivity to the prodrug 5′-DFUR by 165-fold, whereas no difference in the sensitivity to 5-FU was detected. A substantial bystander effect could be detected when transfectants were mixed with the parental MCF-7 cells in vitro.

Deoxycytidine Kinase

Cytosine arabinoside (ara-C) is a cytidine analogue that, when incorporated into replicating DNA, will cause lethal strand breaks. ara-C is an efficient antitumor agent for hematologic malignancies, but has only a limited effect against solid tumors. ara-C requires phosphorylation by deoxycytidine kinase (dCK) to be active. dCK phosphorolates deoxyribonucleosides and thereby provides an alternative to de novo synthesis of DNA precursors [see (96) for a recent review]. It is possible to sensitize glioma cells to ara-C by retro- or adenoviral delivery of the dCK encoding gene (97). dCK transduced glioma tumors implanted either intradermally or intracerebrally showed a significant antitumor effect following ara-C treatment.

E. coli *Guanine Phosphoribosyl Transferase*

The *E. coli* guanine phosphoribosyl transferase (gpt) enzyme has been studied due to its unique dual sensitivity/resistance function. It confers resistance to mycophenolic acid and xanthine, but sensitivity to 6-thioxanthine (6TX) to transfected mammalian cells (98). Transfected sarcoma cells could be selected in vitro for their resistance to mycophenolic acid and xanthine, and treatment of tumors derived from gpt transfected cells with 6TX caused durable tumor regression. Further, retroviral delivery of the bacterial gene to rat glioma cells showed an increased sensitivity of the transfectants and untransfected bystanders to 6TX (99). The bystander effect could be abolished by separating the cells with a microporous membrane, indicating that it was not due to diffusable metabolites. Subcutaneous and intracerebral tumors

grown from the transfected cells could be controlled by 6TX treatment.

E. coli *Nitroreductase*

The nitroreductase from *E. coli* B has been studied for its use in antibody-directed enzyme prodrug therapy (ADEPT). The *E. coli* nitroreductase B, and to a lesser extent the Walker cell Dt-diaphorase, are able to activate CB 1954 [5(-aziridine-1-yl)-2,4-dinitrobenzamide] to a potent interstrand cross-linking agent (100). Nitroreductase is a flavoprotein that requires either NADH or NADPH cofactors, which limits its use for ADEPT. Gene therapy approaches were therefore investigated and transfer of the bacterial gene to V79 hamster cells increased their sensitivity to CB 1954 by 770-fold compared to untransfected controls (101).

Clostridium acetobutylicum *Electron Transport Enzymes*

Metronidazole is the drug of choice for many serious anaerobic bacterial infections. Its use is based on the ability of most bacteria to convert metronidazole to a toxic species, whereas mammalian cells, even under anoxic conditions, lack this ability. Even among bacteria there is a substantial variation in sensitivity. For example, the obligate anaerobe *Clostridium acetobutylicum* is highly sensitive to metronidazole, whereas the facultative anaerobe *E. coli* is up to 500-fold more resistant to the drug. In *C. acetobutylicum* metronidazole is reduced by enzymes of the electron transport system, including the flavodoxin and hydrogenase enzymes (102–104). We are investigating whether the introduction of the bacterial genes coding the flavodoxin and/or hydrogenase enzymes into human tumor cell lines could increase their sensitivity to metronidazole.

The introduction of the Clostridium genes encoding the enzymes flavodoxin or hydrogenase increased sensitivity to metronidazole in *E. coli* by up to 10-fold. Because nitrate reductase-negative *E. coli* cells are already sensitive to metronidazole this is a significant increase in drug sensitivity. The minimal coding regions of the hydrogenase and flavodoxin encoding genes were then introduced into the two human tumor cell lines HT1080 (fibrosarcoma) and HT29 (colon carcinoma) under the control of the strong constitutive CMV promoter. RNA studies showed that the bacterial genes were transcribed in the transfected human tumor cells. However, only a modest increase in metronidazole sensitivity in the human cells was observed (two- to threefold). It is not yet known if a functional enzyme or a protein is produced, but it appears that inefficient translation might be to blame. The regulatory sequence [Kozak sequence (105)] around the start codon differs greatly between the bacterial genes and mammalian genes. We have therefore used site-directed mutagenesis to improve translation and are currently testing the transfectants.

CONCLUSION

By carefully choosing a strategy involving targeted delivery and expression of a therapeutic gene, selective and specific tumor kill in preclinical studies is possible. Gene therapy has the potential to have few side effects and a much lower systemic toxicity than current therapies. It can also selectively target micrometastatic deposits, which are currently difficult to detect or treat. However, choosing only one criteria for selectivity, such as targeting delivery to proliferating cells or tissue-specific expression, is not sufficient, as nonspecific toxicity has been reported. Only by combining the most successful strategies in cancer gene therapy approaches will a successful clinical treatment emerge.

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